LABORATORY TECHNIQUES

FOR VETERINARY TECHNOLOGISTS IN STATE–FEDERAL COOPERATIVE LABORATORIES

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PREFACE

This material has been prepared as a guide of laboratory techniques to broaden the scope and ability of veterinary technologists in State-Federal Cooperative Laboratories.

Only those techniques described for the Brucellosis Tube Test, Brucellosis Rapid Plate Test, and Viability Determination of Strain 19 Vaccine are approved by the Animal Disease Eradication Division, ARS, for use in its Official programs.

The remaining described techniques are for use in determining the disease status of poultry flocks, and gaining additional information on brucellosis problem herds. These should only be used at the discretion of State and Federal Animal Disease Regulatory Officials.

CONTENTS

LABORATORY TECHNIQUES

ESTIMATION OF VIABLE BRUCELLA ABORTUS CELLS IN STRAIN 19 VACCINE

The following serial dilution method is routinely used by the Laboratory Services, Animal Disease Eradication Division, ARS, for determining the number of viable organisms in Brucella abortus vaccine:

Four Pyrex dilution bottles fitted with rubber stoppers or screw caps are used for making the necessary dilutions. Ninety-nine cc. of sterile one percent peptone solution are added aseptically to the first three bottles. In the fourth bottle only 90 cc. are added.

One cc. of thoroughly suspended vaccine is pipetted into bottle No. 1 containing 99 cc. of a one percent peptone solution and shaken for 1 minute. Serial transfers of 1 cc. aliquots are similarly made to bottles No. 2 and No. 3. After bottle No. 3 has been shaken, 10 cc. are transferred to bottle No. 4 containing 90 cc. of one percent peptone solution and shaken in the same manner described for the first three bottles. Bottles No. 1 and No. 2 are discarded as their dilutions are too low for satisfactory counts.

From bottle No. 3, representing a vaccine dilution of 1:1,000,000, one-tenth cc. is plated on each of two serum potato agar, or Tryptose agar plates. Uniform dispersion is obtained by the use of a platinum or nichrome wire spreader. From bottle No. 4, representing a dilution of 1:10,000,000, one-tenth cc. is also plated in the same manner on four plates. Multiple plates are always made on each dilution to insure maximum accuracy. All inoculated plates are inverted and incubated 96 hours at 37°C. after which each is divided into sections by a wax pencil to facilitate counting.

If a plate inoculated from bottle No. 3 contains 1,200 colonies this would represent a total count of 12 billion viable cells per cc. in the original vaccine. A plate inoculated from bottle No. 4 of the same series should have approximately 120 colonies for a corresponding viability. The average count for all six plates is accepted as the final value of the vaccine.

A standard or control of known viability should always be used with series of tests to check the accuracy of the procedure. The results obtained by this method compare favorably with direct counts made on fresh vaccine in the Helber counting chamber.

A count of not less than 10 billion viable brucella cells per 1 ml. at the time of preparation and not less than 5 billion viable brucella cells per 1 ml. at the end of the dating period is considered satisfactory.

Outlined Procedure

Resuspension

Liquid: Shake 1 minute

Desiccated: Reconstitute, shake 1 minute and transfer to a sterile tube. Sufficient time should be allowed for rehydration of the reconstituted product. This usually requires 2 or 3 minutes after addition of the accompanying vial of diluent.

Dilutions

1.0 ml. of vaccine in 99 ml. of sterile Peptone solution and shake 1 minute - 1-100 dilution.

1.0 ml. of 1-100 dilution in 99 ml. of sterile Peptone solution and shake 1 minute - 1-10,000 dilution.

1.0 ml. of 1-10,000 dilution in 99 ml. of sterile Peptone solution shake 1 minute - 1-1,000,000 dilution.

10.0 ml. of 1-1,000,000 dilution in 90 ml. of sterile Peptone solution and shake 1 minute - 1-10,000,000 dilution.

Inoculation

Place 0.1 ml. of the 1-1,000,000 dilution on each of two serum potato agar or Tryptose agar and disperse over the entire surface of plates No. 1 and No. 2 by means of a sterile cooled spreader.

Place 0.1 ml. of the 1-10,000,000 dilution on each of four serum potato-agar or Tryptose-agar plates and disperse over the entire surface of plates Nos. 1, 2, 3, and 4 by means of a sterile cooled spreader.

Note: The spreader should not be flamed between spreading of the inoculum of plates 1 and 2 of the 1-1,000,000 dilution and plates 1, 2, 3, and 4 of the 1-10,000,000 dilution.

Incubation

Invert and incubate plates for 96 hours at 37.5°C.

Determination of Viable Count

Example: If there are 1,200 colonies on plate No. 1 and 1,290 colonies on plate No. 2 representing the 1-1,000,000 dilution:

1,000,000	=	the dilution factor
10	=	the amount of inoculum (0.1 ml.) x 10 - equivalent
10,000,000		for 1 ml.
1,200	=	the number of colonies on plate No. 1
12,000,000,000	=	the number of viable organisms per ml.

Calculate in the same manner for 1,290 colonies of plate No. 2

If there are 128, 114, 121, and 120 colonies on four respective plates from the 1-10,000,000 dilution:

10,000,000	=	the dilution factor
10	=	the amount of inoculum (0.1 ml.) x 10 equivalent for 1 ml.
100,000,000		
128	=	the number of colonies on Plate No. 1
12,800,000,000	=	the number of viable organisms per ml.

Calculate in the same manner for 114, 121, and 120 colonies of plates 2, 3, and 4.

Determination of the Average and Final Viable Count

Plate No. 1 of the 1-1,000,000 dilution 12,000,000,000 viable cells per ml.

Plate No. 2 of the 1-1,000,000 dilution 12,900,000,000 viable cells per ml.

Plate No. 1 of the 1-10,000,000 dilution 12,800,000,000 viable cells per ml.

Plate No. 2 of the 1-10,000,000 dilution 11,400,000,000 viable cells per ml.

Plate No. 3 of the 1-10,000,000 dilution 12,100,000,000 viable cells per ml.

Plate No. 4 of the 1-10,000,000 dilution 12,000,000,000 viable cells per ml.

$$6\overline{)73,200,000,000}$$

$$12,200,000,000$$

12,200,000,000 or 12.2×10^9 is accepted as the final value representing the number of viable brucella cells per 1 ml. of vaccine. Each dilution should be shaken in a horizontal position by hand or mechanical shaker.

Materials

1. Dilution Bottles

Six ounce bottles of boro-silicate type glass of high resistance and low alkalinity are also satisfactory. Ordinary prescription type bottles (so-called soft glass of high alkalinity) are not suitable for this purpose as they may alter the pH of the diluent.

2. Peptone Solution Diluent

Bacto-peptone	20 gm.
Sodium chloride	10 gm.
Distilled water	2,000 ml.

Mix and sterilize in the autoclave for 30 minutes at 15 pounds pressure (121°C.)

3. Potato-Infusion Agar

Potato-infusion	1,000 ml.
Agar	25 gm.
Peptone, Difco or equivalent	10 gm.
Beef extract, Liebig's or equivalent	5 gm.
Sodium chloride C.P.	5 gm.
Glycerine U.S.P.	20 ml.

A. Sound, raw, well cured potatoes are washed, and then sliced, 250 gms. are then added to 1,000 ml. of distilled water with a minimum exposure to air.

B. The media is then infused 16 hours in a covered container at 60°C. and filtered through a single layer of gauze. This filtrate, made up to original volume with distilled water, constitutes the potato infusion used in the formula.

C. All ingredients are added to the infusion and sufficient heat applied to insure complete solution. For small amounts, glass lined, steam jacketed kettles, or double boilers are used.

D. The medium is adjusted to pH 7.0 - 7.2 with sodium hydroxide and boiled for 5 minutes. If necessary, readjustment is made so that the product will have a final pH of 6.0 - 7.0.

E. Sterilization of the medium is accomplished by steam pressure maintained at a temperature of 121°C. for 35 minutes.

F. The sterilized medium is permitted to stand at room temperature until the precipitate has settled. The clear medium is then decanted in 400 ml. amounts.

G. The medium is then cooled to 50°C. and 30 ml. of normal horse serum is added.

H. The medium is poured into petri plates (20-25 ml. per plate) and incubated for 24 to 48 hours at 37°C. It is then held at room temperature for 24 hours before using.

4. Bacto-Tryptose Agar

To re-hydrate the medium, suspend 41 gms. of Bacto-Tryptose agar in 1,000 ml. of cold distilled water and heat to boiling in order to dissolve the medium completely. Dispense medium in 500 ml. Erlenmeyer flasks and sterilize in the autoclave for 15 minutes at 15 pounds' pressure (121°C). The final pH of the medium will be pH 6.9. The medium should be poured in petri plates (20-25 ml. per plate) and incubated for 24 to 48 hours at 37°C. It should then be held at room temperature for 24 hours before using.

Unless the laboratory has facilities to prepare potato-infusion agar medium using aged northern grown potatoes, it may be advisable to use commercially prepared medium. Considerable experience is necessary in the preparation of potato-infusion media to obtain consistent growth promoting properties. Equally good results can be obtained with Bacto-Tryptose agar which has the advantage of being easily prepared. Discrepancies in viable counts obtained using potato-infusion agar and tryptose agar cannot be fully explained. Certain serial lots of strain 19 vaccine of altered vitality will sometimes give a higher viable count on tryptose medium than on potato-infusion agar medium.

AGGLUTINATION TESTS FOR BRUCELLOSIS

Brucella tube and plate antigens produced by Laboratory Services, Animal Disease Eradication Division, ARS, Beltsville, Maryland, is limited to use in the State-Federal Program for the Brucellosis Eradication Program. In the standard tube test specific amounts of serum are added to an antigen containing 0.045 percent brucella cells by volume. Plate antigen is adjusted to 11 percent cells by volume to agree with the standard tube test. The plate test will agree with the tube test in average results in the standard dilutions adopted by committees of the American Veterinary Medical Association and U. S. Livestock Sanitary Association. Each of these two antigens has been standardized to a definite technique. (Details of antigen production will be supplied on request.) Any material variations from the prescribed techniques in performance of these tests may lead to inaccurate results. Pipettes, rheometers, antigen-delivery apparatus and droppers must be standardized equipment to assure that definite quantities of antigen and serum can be delivered. If these preliminary requirements of the tests are met, the only variables will be in the accuracy of the technician in each detail of the test and in its interpretation. Since these variables include many factors difficult to control, it is improbable that identical results will be obtained in all instances in the comparative testing of a number of samples. These variations should be slight and is usually limited to the variance between a complete or partial agglutination in the same dilution.

Tube Test Method

In testing bovine blood serum for routine diagnostic purposes, the "decimal system of dilutions" is most commonly used. This consists of delivering descending quantities of clear serum into test tubes and adding antigen to each tube to obtain final dilutions of 1:25, 1:50, 1:100, and 1:200. Agglutination in the 1:25 dilution is considered negative and agglutination in the 1:100 dilution, positive; therefore, this range is sufficient for diagnosis.

If the endpoint of agglutination is desired, the "multiple dilution system" is used. This consists of setting up a series of 10 to 12 tubes and preparing two-fold dilutions by serial transfer of serum and antigen.

Procedure for the decimal system of dilutions

Blood serum samples should be clearly numbered. After centrifugation to separate serum from clot, the blood tubes are placed in sequence in a holding block. Racks of test tubes in rows of four each are numbered to agree with serum samples. The first sample is removed from the block. A clean, sterile pipette is inserted midway into the separated clear serum and a quantity slightly greater than that required for the test is withdrawn. The serum is allowed to drop back into the blood tube until the bottom of the meniscus in the lumen of the pipette touches the top graduation for delivery. The pipette is then inserted into the first tube and 0.08 cc. serum delivered at the bottom of the tube. The pipette is withdrawn along the side of the tube to allow serum on tip to be delivered. The pipette is inserted into the second tube and 0.04 cc. serum delivered. The third tube receives 0.02 cc. and the fourth receives 0.01 cc. Further dilutions cannot be made with accuracy by this method. Succeeding samples are handled in like manner until the rack is filled. Pipettes with broken tips should not be used.

With a burette or antigen-delivery machine, 2 cc. of tube antigen are delivered into each tube receiving serum. These dilutions correspond closely to 1:25, 1:50, 1:100, and 1:200 respectively. The racks are gently shaken to insure thorough mixture of serum and antigen. The racks are then placed in an incubator for 40 to 48 hours at 37.5°C. The samples of blood serum are placed in a refrigerator until final reading of tests is made.

Procedure for the multiple dilution system

Racks of test tubes containing 10 to 12 tubes are prepared in a row and properly numbered to agree with the blood samples and the dilutions. In the first tube, 0.16cc. of clear serum is deposited. This tube then receives 4 cc. tube antigen and the remaining each receive 2 cc. A 2 cc. rheometer syringe is then used to withdraw 2 cc. of the serum-antigen mixture from the first tube and deliver it to the second tube. The serum-antigen is thoroughly mixed by withdrawing and replacing the full contents of the rheometer several times. The succeeding dilutions are made by repeating this procedure in the remaining tubes. An excess of 2 cc. of the serum-antigen mixture is discarded from the last tube. The rheometer should be rinsed several times between each sample.

In some instances, as much as 0.4 cc. may be erroneously carried over from one tube to another. This is due to serum-antigen mixture remaining in the needle.

Observations

Agglutination consists of the clumping of brucella cells in the antigen. This results in their gravitation to the bottom of the tube with a resultant clearing of the supernatant fluid. Gentle shaking does not disrupt the floculi. The endpoint of agglutination is referred to as the titer of the serum, i.e. If clearing occurs in the first three tubes only and the floculi remain after gentle shaking, the titer of the serum is 1:100.

Agglutination tests should be read against a dull black background. A strong beam of light should pass through the tubes. Extraneous light should be reduced to a minimum. The degree of agglutination in each of the various dilutions may be classified as complete (+), incomplete (I), or negative (-). The limits of accuracy inherent to the test do not justify a more complex classification for routine testing. Readings are interpreted as shown in Table I.

Necessary equipment for the tube test

- Test tubes of the Wassermann type, 12 mm. x 100 mm. of clear glass and thoroughly cleaned.
- Wire racks to hold test tubes; a convenient size will accommodate 15 tests of 4 to 6 tubes each.
- Blocks for holding blood samples, preferably constructed to accommodate 15 samples in one row. This coordination between blocks and racks simplifies the testing procedure.

- Pipettes, 0.2 cc. capacity, (1/100 graduations), or specially graduated for 0.08, 0.04, 0.02, 0.01, and 0.005 cc. deliveries, respectively. A single, standardized pipette may be used provided it is rinsed thoroughly and blown as dry as possible between tests.

- Rheometer of a 2 cc. capacity, with an attached 2.5 or 3 inch needle of a 14 to 16 gauge.

- Automatic antigen-pipetting machine.

- Incubator for maintaining tests at 37.5°C. for required period.

- Refrigerator for storing blood samples during test period.

Plate Test Method

The plate or rapid test for brucellosis does not represent a direct ratio between serum and antigen. The plate antigen is an arbitrary adjustment of cells, so concentrated that when mixed in definite quantity with serially decreasing quantities of serum, the results in the various dilutions will agree with those of the tube test in dilutions of 1:25, 1:50, 1:100, etc. In experienced hands employing a standard technique, the plate test will provide results comparable with those of the tube test. Occasionally a serum sample may be encountered that will not react in the same degree with the tube test and the plate test. The reason for such variation is not clearly understood. As ARS plate antigen has been adjusted to a definite technique, any deviation from the standard in making the plate test will result in inaccurate results.

Procedure for Conducting the Plate Test

A preliminary screening of samples by the plate method is usually advisable. This usually results in a considerable saving of time and antigen.

Screening may be accomplished by using a single dilution to determine whether the sample is negative or suspicious. A dilution less than 1-50 is used for this purpose, i.e., 0.05 cc. of serum and one drop of antigen gives a 1-42 dilution. If the sample is negative by this method, no further testing is necessary. If it is positive, further testing is necessary to determine the titer.

The actual technique of conducting the screening test and the titering of a sample are alike except for the amounts of serum used and the resulting dilutions, therefore, they will be discussed as one.

The dilutions obtained from different amounts of serum and antigen are as follows:

Serum	Antigen	Dilution
0.05 cc.	1 drop 0.03 cc.	1-42
0.04 cc.	1 drop 0.03 cc.	1-50
0.02 cc.	1 drop 0.03 cc.	1-100
0.01 cc.	1 drop 0.03 cc.	1-200

The light in the testing box should be turned on to warm the plate slightly before the test is begun. Both serum and antigen should be allowed to warm, to meet room temperature. With a serological pipette held at an angle of approximately 45°, the serum is deposited onto the plate. Succeeding dilutions or samples are deposited onto the test plate in the same manner and in a row. The antigen bottle is shaken gently to insure a homogenous suspension. Holding the dropper in a vertical position, one drop of plate antigen (0.03 cc.) is dropped onto each quantity of serum.

The serum and antigen are then mixed thoroughly by use of a spreader. The testing plate is lifted from the box and gently rocked in a rotating movement for further mixing. The plate is replaced and the lid closed. The lights should be turned off to decrease evaporation. Antigen should be kept under refrigeration when not in use to prevent bacterial contamination beyond the inhibiting power of the preservative.

Equipment needed for the plate test

- Testing box.--A satisfactory box is about 17 inches long, 13 inches wide and 5 inches deep. The glass testing plate, etched to make sixty 1-1/2 inch squares in 5 rows of 12 is supported near the top of the box and can be easily lifted from the box; two electric lights are partially encased below the plate near the front of the box so that oblique light may be thrown from beneath toward the serum-antigen mixture; the inside of the box should be painted a dull black; the box should also be provided with a hinged cover to prevent too rapid evaporation of the mixtures. Several commercial test boxes are available for purchase which include all these essential features.

- Pipettes.--The pipettes described for the tube test are satisfactory for the plate method.

- Antigen dropper.--The antigen dropper should be calibrated to deliver exactly 0.03 cc. of antigen per drop. All droppers should be carefully tested before initial use, by delivering from a vertical position, 100 drops of antigen into a 5 cc. graduate. A satisfactory dropper is one that delivers 3 cc. by this method. A 13 gauge needle, 2-1/2 inches in length, from which the tapered end has been cut off evenly and the opposite end equipped with a soft rubber bulb, makes a satisfactory dropper after calibration of dropping end.

- Spreader.--Toothpicks may be used for spreading the serum and antigen, however a strong wire bent to obtain a straight one-half inch bearing surface is more satisfactory. A multiple spreader can be made to spread similar dilutions in four tests simultaneously. The spreader should be dipped in water and wiped dry between samples.

Observations

The tests should be allowed to incubate for 8 minutes before observations are made. Most samples reach their peak of agglutination in that time. All samples should be incubated at room temperature, 72°F to 78°F, to maintain the greatest degree of uniformity. Caution should be used to avoid prolonged heating of the plate with incandescent lights.

The plate should be removed from the box and gently rotated after approximately 4 minutes of incubation. After a total of 8 minutes incubation, the lights should be turned on and the box tilted slowly. This will allow the mixture to flow from side to side while readings are being made. Observations are best made against the dull black background of the box. Only three classifications of reaction should be made, complete (+), incomplete (I), and negative (-). Readings are interpreted as shown in Table I. All tests on the plate should be read within 1 minute in order to maintain uniformity of test procedures and proper incubation.

TABLE I.--Interpretation of Reactions

Dilutions				Diagnosis	
1:25	1:50	1:100	1:200	Non-vaccinated Cattle	Vaccinated Cattle
-	-	-	-	Negative	Negative
I	-	-	-	Negative	Negative
+	-	-	-	Negative	Negative
+	I	-	-	Suspicious	Negative
+	+	-	-	Suspicious	Negative
+	+	I	-	Suspicious	Suspicious
+	+	+	-	Reactor	Suspicious
+	+	+	I	Reactor	Suspicious
+	+	+	+ or higher	Reactor	Reactor

THE ACIDIFIED PLATE AGGLUTINATION TEST FOR BRUCELLOSIS

The acidified plate agglutination test, is a sero-agglutination test using the plate antigen to which a prescribed amount of acid has been added so that the resulting mixture of antigen and serum will have a designated pH.

When 0.04 ml. of 85 percent lactic acid (reagent grade) is added to 1 ml. of plate antigen, the resulting mixture of one standard drop (0.03 ml.) with 0.08 ml. of serum will have a pH of 3.8.

In conducting the acidified plate agglutination test, only the 0.08 ml. of serum or 1/25 dilution is used. Mixing of the antigen and serum should be thorough. The plate is rotated for mixing at 8 minutes and just before reading of the test at 15 minutes. With the appearance of any agglutination reaction, the sample should be considered positive.

For additional information see references 13 and 14.

METHOD OF CONDUCTING A HEAT INACTIVATION BRUCELLA TUBE AGGLUTINATION TEST

The heat inactivation test is a method which is intended to differentiate between specific or nonspecific agglutinations. The efficiency of the test in differentiating between these is not clear and experimental work is in progress by workers in the field.

The test is conducted in a manner similar to the blood serum tube agglutination test except that the tubes are incubated in a thermostatically controlled waterbath at 56°C. for 16 to 18 hours.

The reading of the test results is the same as the tube agglutination test.

For additional information see references 8 and 11.

WHEY TEST FOR BRUCELLOSIS

The whey test is a method of detecting brucella agglutinins by using the serum fraction of milk. The Cameron whey test 1, 2, 3, 4, is conducted as follows:

1. Add 2-3 drops of rennet to approximately 10 ml. of milk, mix and incubate for 1-2 hours at 37°C. or overnight at room temperature.

2. The test is then conducted in a manner similar to the blood serum plate test using 0.08, 0.04, 0.02, and 0.01 ml. of whey mixed with one standard drop (0.03 ml.) of Brucella Ring Test (B.R.T.) antigen.

3. The plate is rotated after mixing and again at 8 minutes. Reading of the test is made after 10 minutes of incubation at room temperature.

4. Reading of the test is as follows: No agglutination = 0, agglutination in the 0.08 amount of whey = 1, 0.04 = 2, 0.02 = 3 and 0.01 = 4.

   The interpretation of the readings is as follows:

   0 & 1 = negative
   2 = suspicious
   3 & 4 = positive

For additional information see references 3, 4, 5, and 6.

TESTING PROCEDURES FOR PULLORUM DISEASE-- THE STANDARD TUBE AGGLUTINATION TEST*

The blood samples shall be collected by a properly qualified and authorized person. Suitable blood tubes, shipping containers, and bleeding and leg-banding equipment should be furnished by the serological laboratory or the authorized agency in charge of

*Taken from "Diseases of Poultry" by Biester & Schwarte, 3rd Edition.

the testing program. Blood tubes should be thoroughly cleaned and heated in a hot-air sterilizing oven. Cork stoppers should be boiled or washed and dried in a hot-air drying oven. Shipping containers for the blood samples should be constructed to permit washing and disinfection.

All birds tested are to be officially leg-banded. The blood tube is identified with the leg-band number which is inscribed on the etched portion of the tube or on a gummed label. A small amount of blood (1/2 to 2 cc.) is collected from the median vein of the wing (Vena cutanea ulnaris) by incising the latter with a sharp-pointed lancet or knife. The tube is laid on its side permitting the blood to clot in a long slant. After the blood has coagulated, the samples are packed in containers designed for shipment by mail, express service, or special messenger to the laboratory. In extremes of temperature, precautions should be taken against freezing or overheating because the blood samples should arrive at the laboratory in a fresh state and unhemolyzed condition for a satisfactory test. All hemolyzed or spoiled samples should be rejected. The diagnostic laboratory should be equipped with proper and adequate refrigeration facilities where blood samples should be retained until the sera have been tested and results of the tests are known. Occasionally, a retest on the same serum may be necessary to determine the pullorum status of a bird.

Preparation of Antigen

The antigen for the tube test should be prepared from representative strains of S. pullorum which are known to contain the different antigenic components normally found in S. pullorum. Furthermore, the strains should possess high agglutinability with positive serum but should not agglutinate with negative or nonspecific sera. Stock cultures of the antigen strains should be grown and maintained on nutrient agar medium composed of dry granular agar (Difco) 2.0 percent, Bacto-Peptone (Difco) 1.0 percent, beef extract (Difco) 0.4 percent and water. The final hydrogen-ion concentration should range from 7.0 to 7.2. The cultures should be transferred not more than one a month. Seed cultures should be taken from the stock strains rather than from rapid serial transfers in order to avoid contaminants or possible variation in the characteristics of the organism. Large test tubes, Kolle flasks, or Blake bottles containing nutrient agar medium may be used for producing the antigen. After 48 to 72 hours' incubation, the growth is washed off with sufficient phenolized (0.5 percent) saline (0.85 percent) solution to produce a very concentrated suspension. This suspension is filtered through sterile absorbent cotton or glass wool into sterile glass-stoppered bottles. The washings for each of the three strains are combined in equal volume-density, and the stock antigen is stored at 8 to 10°C.

Dilution of Antigen for Testing

For routine testing, a dilute antigen is prepared from the stock antigen by diluting the latter with physiological saline solution containing 0.25 to 0.3 percent phenol. The turbidity of the antigen corresponds to 0.75 - 1.00 on the McFarland Nephelometer scale, and the hydrogen-ion concentration is adjusted to pH 8.2 - 8.5 by the addition of dilute sodium hydroxide. The dilute antigen is prepared each day in order to reduce dissolution and plasmolysis to a minimum at the specified hydrogen-ion concentration.

Setting up the Test

The amount of diluted antigen employed in individual tests may vary from 1 to 2 cc; however, the amounts should be constant and placed in clean, clear test tubes. Commercial devices are recommended for this phase of the work. The sera are added to the test tubes containing the antigen with a serologic pipette or a serum-delivery device which is accurately calibrated to deliver definite amounts. The maximum dilution employed must not exceed 1:50 and, according to available data, the 1:25 dilution appears to be the most efficient. After the serum and antigen are well agitated, the mixture should be incubated for at least 20 hours at 37°C.

Reading the Tests

The results of the tests are interpreted as follows:

- A negative reading represents a test in which the fluid remains uniformly turbid.
- A positive reading represents a test in which the antigen reveals a distinct clumping, and clumps of cells have settled to the base of the tube with the supernated fluid being clear.
- Suspicious test - Graduation of clumping or agglutination may occur between negative and complete positive tests. These may be designated as slightly and strongly suspicious.

Reporting Test Results

All suspicious and positive reacting tests should be reported to the agency responsible for the disposition of infected birds. Also, all broken, missing, and spoiled samples should be reported. In case the past status of the flock has been free of infection and only a few reactors are detected, the serologic diagnosis should be confirmed by bacteriologic examination of the reactors. Such a procedure will avoid a false diagnosis of fowl typhoid or paratyphoid infections. If only suspicious reactions are observed in a flock, then the strongest reacting birds should be submitted to the laboratory for retesting and a careful bacteriologic examination. In routine testing, flocks should not be condemned as infected on the basis of doubtful or typical reactions because such reactions may be due to causes aside from S. pullorum. If no conclusive evidence of pullorum infection can be found, the flock should be regarded as negative. This statement is based on observations made in routine testing in the New England States during the past decade. The lowering or removing of the official pullorum status of a flock should be exercised only after conclusive evidence of infection has been established.

The Rapid Serum Test

The rapid serum test for the detection of pullorum diseases carriers was developed in 1927. The blood samples may be collected in a manner similar to that described for the tube test. The antigen employed should consist of representative strains of S. pullorum which are of known antigenic composition and high agglutinability, but which are not sensitive to negative and nonspecific sera. The strains are suspended in 12 percent sodium chloride solution containing 0.5 percent phenol. The turbidity is adjusted to 50 times greater than tube 0.75 of McFarland's nephelometer.

A box with a glass top ruled off in inch squares and improvised with lighting and heating facilities was used for testing. Two serum-antigen dilutions corresponding to the 1:50 and 1:100 dilutions for the tube test were employed. The amounts of serum used were 0.02 cc. and 0.01 cc. to which was added 0.02 cc. of antigen. The serum and antigen were mixed thoroughly with a toothpick. Positive reactions may occur quickly, but delayed reactions may require several minutes. Graduations of reactions occur in this method as in other methods. Considerable experience is necessary for proper interpretation. This method should be used only in competent hands if the results are to be regarded as official. The results of the tests and the numbers of spoiled, broken, and missing samples should be reported directly to the flock owner or the agency in charge of the field work.

TESTING SERVICE OUTLINE FOR CARRIERS OF SALMONELLA TYPHIMURIUM INFECTION

The macroscopic tube agglutination test at a 1:25 dilution (i.e. 0.04 cc. of serum and 1 cc. of diluted antigen) is used for conducting the test. Serological tubes having the dimensions of 100 x 13 mm. are preferable for setting up the test. Two antigens are employed to test each serum sample. The first is a somatic of "O type" antigen prepared from the body of the bacterial cell; the second is a flagellar or "H type" antigen prepared from actively motile forms of the organism and containing a well-balanced mixture of both phase 1 and phase 2 antigenic factors. It is best to employ both the O and H antigens since some birds carry agglutinins for only one or the other antigen and thus may not be detected if only a single antigen is used.

Preparation and Storage of the Diluted Antigen

The antigens are diluted in accordance with directions on the label. Antigens should be diluted immediately before use and only that amount to be employed in the tests should be diluted at any one time. The concentrate should be stored under refrigeration at all times. The pH of the diluted antigen suspensions need not be adjusted before use.

Incubation of Tests

The O tests are incubated in the regular bacteriological incubator at 37°C., and read after a full 24 hours of incubation. Positive reactors are indicated by a granular type agglutination.

The H tests are incubated preferably in a water bath set at 50°C., and read after 2 hours incubation. If no water bath facilities are available the tests may be incubated in the regular bacteriological incubator at 37°C., and read after a full 24 hours of incubation. When the water bath incubation method is used, the tubes should be allowed to set at room temperature for 15 minutes before readings are made. The tubes should not be subject to agitation previous to reading since the H agglutination is very floccular and loosely bound. Only gentle agitation should be used in making the readings.

Retests of Flock Revealing O Reactors

When the O antigen is used as the "finding test" and reactors are encountered, the entire flock should be retested using H antigen. This is necessary to insure that all

reactors are detected. As far as possible, both the O and H antigens should be used in testing all flocks on initial tests.

Titration of Reacting Serums

Serums reacting to either the O and H antigen at the 1:25 dilution should be titered. Six dilutions should be used according to the following protocol:

Tube No.	0.85% Aqueous NaCl	Serum: Preparation of Dilution	Antigen (diluted)	Final Dilution
1	0.9 cc.	0.1 cc. of serum*	0.5 cc.	1:20
2	0.5 cc.	0.5 cc. from tube No. 1	0.5 cc.	1:40
3	0.5 cc.	0.5 cc. from tube No. 2	0.5 cc.	1:80
4	0.5 cc.	0.5 cc. from tube No. 3	0.5 cc.	1:160
5	0.5 cc.	0.5 cc. from tube No. 4	0.5 cc.	1:320
6	0.5 cc.	0.5 cc. from tube No. 5	0.5 cc.	1:640

*The contents of each tube should be thoroughly mixed by sucking up the fluid in the pipette and blowing it back into the tube several times before transferring the 0.5 cc. to the next tube. After mixing, 0.5 cc. is discarded from tube No. 6 before addition of the antigen. A 1 cc. pipette graduated to tip is the most convenient size. Tubes of about 13 mm. inside diameter are suitable for this volume of fluid.

Dilution of Antigens for Titrations

In diluting the antigen for setting up the titrations as outlined above the amount of diluent indicated on the label of the concentrates should be divided by one-half. For example, if the regular dilution factor for the antigen is 1 cc. of concentrate to 20 cc. of diluent, then this should be changed to 1 cc. of concentrated antigen to 10 cc. of diluent. Also, the phenol or formalin concentration should be doubled in the diluent (i.e. 0.5 percent in place of 0.25 percent) to provide a final preservative concentration of 0.25 percent.

Titration Using Undiluted Serum

Serums may also be titrated by using the undiluted serum added in serially decreasing amounts to 1 cc. of antigen diluted in the usual manner according to the following protocol:

Tube No.	Serum	Antigen (diluted according to label)	Final Dilution
1	0.04 cc.	1 cc.	1:25
2	0.02 cc.	1 cc.	1:50
3	0.01 cc.	1 cc.	1:100
4	0.005 cc.	1 cc.	1:200

This method has the disadvantage of limiting the number of dilutions that can be conducted.

Final Serological Tests on Reactors

When birds are submitted to the laboratory the following serological tests should be conducted before the birds are slaughtered for culture:

- Rapid whole-blood test with stained S. typhimurium antigen. One loopful of blood and one drop of antigen should be used as in the pullorum test. Reading time of the test should be limited to 1 minute.

- O tube agglutination test. A fresh serum sample should be collected and set up in six serial dilutions, incubated at 37°C. and read after 24 hours.

- H tube agglutination test set in six serial dilutions as for the O tests, incubated at 50°C. for 2 hours or 37°C. for 24 hours where water bath facilities are not available.

For additional information see references 1, 2, 7, 9, 10, 12, and 17.

PLATE AGGLUTINATION TEST FOR LEPTOSPIROSIS

A practical agglutination test for the detection of leptospirosis, employing a stable formalin-killed antigen, has been developed and known as the Stoenner method or plate test.

A survey made with blood samples received at the brucellosis testing laboratories consists of four distinct techniques:

- Pooling individual samples to make a pooled sample.

- Screening of the pooled sample.

- If the screen test of the pooled sample is negative no further work is necessary on dividual samples represented in such pool. If the screen test on pooled samples is positive it is then necessary to determine the offending individual samples by screening each individual sample represented in the pool.

- Titration, using fourfold final dilutions of serum to determine titers of individual reacting samples.

The plate agglutination test is considered safe for the operator as the antigen employed is formalin treated. The antigen retains a high degree of specificity over long periods of time when properly handled.

Procedures

Pooling

Herd samples are selected following completion of the brucellosis test. Instead of screening all individual animal sera from a herd, it is recommended that clear sera from groups of 10 animals or less be pooled into a clean tube. The pooled sample may

then be screened. The pooling can be easily performed with a micro-pipette by setting the adjustment on the pipette to pick up a 0.2 ml. aliquot from each sample i.e., when a herd of 50 animals is selected, only five pooled samples will be screened. If reactions are found in any of the pooled samples, it will be necessary to screen each sample in the lot to determine which sample or samples are the offending samples. Once selected, they are set aside for titration.

CAUTION: The micro-pipette should be rinsed and flushed between groups of 10 samples, or one pool to prevent carry-over and unnecessary retesting. Clean warm water may be used for this purpose.

Screening

The screening of a pooled sample and the individual samples represented in a positive pooled sample can be accomplished as follows:

A culture loop can be used for making routine screening tests. A nichrome wire loop, 3 mm. in diameter and of 24 gage, (formed around a sixpenny finish nail) will deliver .005 to .006 ml. Some technicians prefer using the brucellosis pipette delivering .005 ml. (last graduated volume) for the screen test. If a wire loop is used, it should be slightly bent at the end so that only the loop will touch the serum surface in the vial, when held at a desired angle. When transferring a loop full of sera, the loop should be touched on the plate and lightly tapped several times in order to deposit the sera. The loop should be dipped in clean hot water, tapping the wire on the edge of the vessel thus freezing it of excess water prior to reuse. A flame may be used to dry the loop after rinsing and prior to use. If this method is used precaution is necessary to avoid using a hot loop in the serum to be screened. As a routine procedure, 10 to 12 sera should be plated at one time. A drop of 0.03 ml. of antigen is added to each test. This is mixed with the serum into a small droplet approximately 1/2-inch in diameter. The plate should then be rotated for mixing and incubated for 6 minutes at room temperature (approximately 75^{o}F.) in a dust-free area. The second group of 10 or 12 samples may then be set up for screening. This procedure of setting up and reading the tests can be alternated as time will permit.

CAUTION: The antigen is stable but subject to electrolytic action of materials other than glass, rubber, or stainless steel. Contact with these materials may cause auto-agglutination and cause clouding or precipitation of the antigen. A small manual-bulb dropping bottle with a stainless steel or glass dropping tube, calibrated to deliver 0.03 ml. in each drop should be used. It is advisable to transfer 10-15 ml. of antigen from the stock 60 ml. bottles and use in such a manner as to protect the antigen. The screen test is conducted in a low dilution, therefore, it is necessary that further titrations be made to properly classify the reaction. (see interpretation - page 18.)

In preforming the screen test, observation of the plate following 15 rotations will usually locate the stronger reactions. The plate should then be rotated another 15 times or an additional 30 seconds. This second rotation may disclose additional sera showing reactions. The reactions are more difficult to see than in the brucellosis test, i.e., the agglutinations are quite fine, appearing only at the periphery as a silvery ring or "halo". Debris, contamination, and dust particles will float in the center of the test and should not be confused as reactions. A negative test is characterized by its slight homogenous opalescence.

The light in the brucellosis test box should be intensified by placing an aluminum foil reflector behind the bulbs. The cover over the light compartment should be opened slightly to permit the concentrated light rays to reach the under side of the agglutination plate. Light should reach the plate at an oblique angle, yet shielded from the operator's eyes.

Titration of Samples by the Plate Method

The titer determination is more time-consuming, therefore, the pooling and screening techniques are extremely valuable in locating sera with reactions. This procedure may be used directly on samples from highly suspicious animals. Titers may be determined by the rapid plate test in a manner similar to that described for the screen test, or by the capillary tube method. In either method fourfold dilutions of serum should be made prior to the actual test.

Normal saline should be used to make four dilutions of the serum. Test tubes 10 mm. x 75 mm. or 12 mm. x 75 mm. are recommended for making these dilutions. These tubes should be placed in a rack in front of the serum to be tested. By use of a Cornwall pipetting syringe delivered 0.8 ml. of saline into the first row of tubes. The remaining three tubes or three rows of tubes should each receive 0.6 ml. of saline. A 1:5 dilution is then made by adding 0.2 ml. of unknown serum and mixing with the 0.8 ml. of saline. This is best accomplished with a 1 ml. serological pipette (1/100 graduations). Draw up the mixture and expel the same several times to thoroughly mix the dilution. The first tube will then contain a 1:5 dilution. With the continued use of the 1 ml. pipette (1/100 graduations) draw up 0.23 ml. of the dilution. Immediately deposit 0.03 of the dilution onto the desired area of plate and continue to transfer the remaining 0.2 ml. to the second dilution tube. This tube contains 0.6 ml. of saline and will make a 1:20 dilution. Draw up the mixture of 1:20 dilution and expel it several times for thorough mixing. Again withdraw 0.23 ml. of the dilution and deposit 0.03 ml. to the second area of the plate. This procedure is repeated until the four dilutions of serum have been made and deposited on the plate. Upon the addition of an equal amount of antigen, 0.03 ml. the final dilutions becomes 1:10, 1:40, 1:60, and 1:640.

Note: The technician will soon learn the time limits of this operation. Usually a group of five unknown serum samples can be diluted, antigen added, rotated, and placed in incubation in approximately 3 minutes. Immediately set up the next group of five samples, each with four dilutions and incubate. It will be found that both groups of five tests should be read before attempting to proceed with further testing. A separate 1 ml. pipette should be used for each sample. If the pipette is rinsed twice in clean water, then blown free of liquid, it may be used throughout the testing procedure. Tests for titer determinations should be rotated 30 times before reading.

Titration of samples by the capillary tube method

This method has some advantages over the plate titration method i.e. where large volume of titration are made or where screen-positive serum samples are found on daily work and are refrigerated and accumulated for delayed titer determinations at specified intervals.

Fourfold serial dilutions are again made by adding 0.2 ml. of unknown serum with 0.8 ml. saline. The subsequent dilutions are made by a serial transfer of 0.2 ml. of the

1:5 dilution to the second tube containing 0.6 ml. of saline to make a 1:20 dilution. The process is repeated to make the 1:80 and 1:160 dilutions. The dilutions of sera are made first, with the tests being made later in bulk lots. When all dilutions are made, capillary tubes (68 mm. x 0.8-1.1 mm. ID) are filled 1/3 full of antigen. The tubing should be held at a low angle. A small open container holding a limited amount of antigen will be found advantageous. An equal amount of the serum dilutions should then be introduced into the capillary tube. This combination of antigen-serum dilution thus fills the capillary tube 2/3 full. The index finger, over the open end of the capillary tube, controls the filling. After this is accomplished, invert the capillary tube and allow the column to shift so that an equal air space exists at both ends.

Return the capillary tube to an upright position and place each respective test into a block containing plastisene or modeling clay.

These tests can be placed in the order of ascending dilutions, within close proximity and at an angle of approximately 70°. The number of the unknown serum should be marked on the block for proper identification. Only a small unused area need be left between the dilution tests of "one each" sample. A pine block, 12 inches long, 2-1/2 inches wide, and 3/4 inch thick with a routed grove is used. This is filled with modeling clay and will hold the dilution tests of 10 to 12 animals.

When a group of capillary tests are set up, they should be incubated overnight at $37.5^{\circ}C$. The following morning the tests should be refrigerated 15 to 20 minutes. When removing the tube from the refrigerator, wipe the lower side of the tubes with a dry cheese cloth to remove vapor condensation and fingerprints. The tests may then be read. A positive reaction is represented by agglutination appearing within the capillary tube. The aid of a strong light will facilitate in the reading. The highest dilution, showing positive agglutinations is considered the endpoint titer regardless of the degree of reaction.

Interpretation of the Plate and Capillary Tube Tests

The following interpretation is recommended as a classification of sero-reactions:

- Herds showing no evidence of blood titers on the screen test should be classified negative. Monthly reports should show the number of herds and cattle.

- Herds showing only a few reactions on the screen test with none exceeding endpoints of 1:40 are considered suspicious herds. The monthly report should show number of herds, number of negative animals, and the number of suspicious animals i.e., screen-positive but limited to 1:40 titers or less.

- Herds showing reactions of 1:160 or greater are considered positive. In this type of herd, those animals showing reactions of 1:40 are considered positive. The monthly report should show number of herds, number of negative cattle, number of suspicious animals (screen-test-positive but no reactions higher than 1:10) and the number of reactors (1:40, 1:160, and 1:640.)

For additional information see references 15 and 16.

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